Leptospirosis pulmonary haemorrhage syndrome is associated with linear deposition of immunoglobulin and complement on the alveolar surface

J. Croda[1], A. N. D. Neto[1], R. A. Brasil[1], C. Pagliari[1], A. C. Nicodemo[2] and M. I. S. Duarte[1]

1) *Laboratory of Pathology of Transmissible Diseases, Department of Pathology, Hospital das Clínicas and* 2) *Infectious and Parasitic Diseases Division, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil*

Abstract

Leptospirosis is a zoonotic infection associated with severe diseases such as leptospirosis pulmonary haemorrhage syndrome (LPHS). The cause of pulmonary haemorrhage is unclear. Understanding which mechanisms and processes are involved in LPHS will be important in treatment regimens under development for this life-threatening syndrome. In the present study, we evaluated 30 lung specimens from LPHS patients and seven controls using histology and immunohistochemistry (detection of IgM, IgG, IgA and C3) in order to describe the pathological features associated with this syndrome. Immunoglobulin deposits were detected on the alveolar surface in 18/30 LPHS patients. Three staining patterns were observed for the immunoglobulins and C3 in the lung tissues of LPHS patients: AS, delicate linear staining adjacent to the alveolar surface, which was indicative of a membrane covering the luminal surface of type I and II pneumocyte cells; S, heterogeneous staining which was sporadically distributed along the alveolar septum; and IA, weak, focal intra-alveolar granular staining. Human LPHS is associated with individual and unique histological patterns that differ from those of other causes of pulmonary haemorrhage. In the present study, it was found that the linear deposition of immunoglobulins (IgA, IgG and IgM) and complement on the alveolar surface may play a role in the pathogenesis of pulmonary haemorrhage in human leptospirosis.

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Corresponding author and reprint requests: M. I. S. Duarte, Universidade de São Paulo, Faculdade de Medicina, Departamento de Patologia, Av. Dr. Arnaldo, 455 - 1° andar, sala 1115, Cerqueira César, São Paulo, São Paulo 01246-903, Brazil
E-mail: miduarte@usp.br

Introduction

More than 500 000 cases of leptospirosis are estimated to occur worldwide each year [1]. Leptospirosis is an environmentally transmitted zoonotic disease. The principal mode of transmission is contact with water or soil contaminated with urine from animals infected with the *Leptospira* bacterium [1–3]. Infection entails a broad spectrum of clinical manifestations, ranging from self-limiting febrile illness to Weil's disease, which is the classic severe form of the disease, characterized by jaundice, acute renal failure and bleeding. The Nicaraguan outbreak in 1995 raised awareness of the importance of leptospirosis as a cause of a pulmonary haemorrhage syndrome [4]. Reports from Asia and the Americas now indicate that leptospirosis has become a major cause of haemorrhagic fever [5–10]. The fatality rates linked to the leptospirosis pulmonary haemorrhage syndrome (LPHS) are >50% [6,7,10].

There is an urgent need to improve therapy and supportive care for patients with LPHS. Novel therapies such as plasma exchange and corticotherapy may be beneficial [11,12]. Therefore, increased understanding of the disease pathogenesis is crucial. It is generally believed that neither thrombocytopenia nor the decrease in clotting factors in leptospirosis patients is sufficient on its own to account for the observed bleeding diathesis [13–16]. Histological and electron microscopy studies in human and guinea pig models were unable to demonstrate significant intravascular thrombi and small vessel fibrin deposition [13,16,17].

Nally *et al.* reproduced the LPHS in a guinea pig model [16] using *Leptospira interrogans* serovar Copenhagen strains

isolated from Brazilian patients who died from haemorrhage and acute respiratory failure [18]. This study demonstrated that immunoglobulin and complement were deposited along alveolar septa and that it could be associated with pulmonary haemorrhage [16]. Also, immunoglobulin deposition on the alveolar septum and alveolar space were observed in a human case of LPHS [19]. However, other than in this single case report [19], these findings have not been reported in larger case series nor, as this one case suggests, has it been established whether an autoimmune process does indeed contribute to the pathogenesis of LPHS. Understanding whether such processes found in experimental animals occur in humans will be important in developing treatment regimens for this life-threatening syndrome. In the present study, we examined lung samples from 30 patients who died of LPHS in Brazil, using histology and immunohistochemistry (IHC) to determine whether the presence of immunoglobulin and complement deposits on the alveolar surface was associated with this syndrome.

Patients and methods

The records of the Death Verification Service from Department of Pathology, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo were reviewed to identify all deaths as a result of LPHS which occurred between 1 January 1988 and 1 January 2005. Confirmation of leptospirosis involved the following criteria: a > four-fold rise in the microagglutination titer; a single titer >1 : 400; and/or detection of *Leptospira* in biopsy or autopsy specimens during immunohistochemical analysis.

In addition to analysis of autopsy specimens from LPHS cases, autopsy specimens were evaluated as controls from seven patients with pulmonary haemorrhage which was confirmed to be a result of other diseases (two patients with acute pulmonary oedema associated with heart failure, three patients with disseminated intravascular coagulation associated with sepsis and two patients with cirrhosis). Controls were randomly selected from the records of the Death Verification Service of Sao Paulo state.

Information on demographics, past medical history, clinical presentation, laboratory findings and outcome was extracted from the medical records and computerized laboratory records using a standardized data entry form.

Lung tissue samples were fixed in neutral, buffered formaldehyde (4%) (Medical Chemical Corporation, Torrance, CA, USA) and processed according to standard methods which included embedding in paraffin, sectioning into 4-μm serial sections and staining with haematoxylin and eosin (H&E). Lung sections were processed for immunohistochemical analyses using polyclonal antibodies. Antibody binding and visualization were carried out using the streptavidin-biotin peroxidase method, with an endogenous biotin blocking system (DAKO, Carpinteria, CA, USA). The following polyclonal antibodies were used in this process: polyclonal rabbit anti-human IgA (A0262/Dako), polyclonal rabbit anti-human IgM (A0425/Dako), polyclonal rabbit anti-human IgG (A0423/Dako) and polyclonal rabbit anti-human C3C (A0062/Dako). The same protocol was used to detect *Leptospira* antigens with a polyclonal rabbit antibody [17,20,21].

Initially one observer (J.C.) analysed six paired H&E- and IHC-stained lung sections to determine the principal features related to LPHS. Subsequently, the 37 (30 LPHS patients and seven controls) sections were analysed by one independent pathologist blinded to patient identity, diagnosis and clinical course (R.A.B) using semi-quantitative scores: 0 = absence; 1 = mild; 2 = moderate; and 3 = intense. In the alveolar space and the alveolar septae, the observers scored the sections for macrophages, lymphocytes, neutrophils, plasma cells and eosinophils. In the alveolar wall and lumen, they scored the sections for necrosis, regeneration of pneumocytes, giant cells, oedema, haemorrhage and hyaline membranes. The sections were also scored for septum thickness, oedema, capillary congestion and fibrosis. The pathologist scored the following findings based on standardized scoring protocols previously used [22].

Data were entered into EPIDATA (version 3.0; U.S. Centers for Disease Control and Prevention) and analysed using the SAS system (version 9.1; SAS Institute, Cary, NC, USA). Results are expressed as the median and interquartile range, or as percentages when appropriate. Differences in frequency were evaluated using the χ^2 test or Fisher's exact test. Medians were compared using the Mann–Whitney–Wilcoxon test. The study protocol was approved by the Investigational Review Board of the Hospital das Clínicas, São Paulo University and the institutional ethical guidelines were followed.

Results

During the study period, we identified necropsies from 37 LPHS patients. Necropsies from seven patients were excluded because the paraffin blocks showed evidence of tissue autolysis, there was inappropriate fixation with artefacts or there was typical bacterial infection. This selection resulted in a final sample of 30 leptospirosis patients who presented with well-defined clinical and histological evidence of lung involvement.

TABLE 1. Clinical and laboratory findings for patients with leptospirosis pulmonary haemorrhagic syndrome

Clinical characteristics	
Age, median (IR)	48.5 (40–56)
Male gender, *n*(%)	23/30 (77%)
Duration of symptoms, median (IR)	6 days (4–7)
Clinical symptoms on admission	
Fever, *n*(%)	26/26 (100%)
Muscle pain, *n*(%)	23/24 (96%)
Headache, *n*(%)	17/23 (74%)
Nausea or vomiting, *n*(%)	13/24 (54%)
Diarrhoea, *n*(%)	6/6 (25%)
Laboratory values	
Haematocrit %, median (IR)	26 (20–30)
Leucocytes, cells/mm^3,median (IR)	1.6×10^4 ($1.1–1.9 \times 10^4$)
Platelets, cells/mm^3,median (IR)	5.4×10^4 ($3.6–10.0 \times 10^4$)
Serum creatinine, mg/dL, median (IR)	6.1 (4.0–7.6)
Serum bilirubin, mg/dL, median (IR)	18.7 (10.6–25.0)
AST, mg/dL, median (IR)	105 (51–221)
ALT, mg/dL, median (IR)	77 (31–142)
Prothrombin time (INR), median (IR)	1.35 (1.00–1.40)
Partial thromboplastin ratio, median (IR)	1.1 (0.9–1.5)
Clinical course	
Use of vasoactive drugs, *n*(%)	22127 (81%)
Duration of mechanical ventilation, median (IR)	2 day (0–5)
Dialysis, *n*(%)	19127 (70%)
Duration of hospitalization, median (IR)	3 days (1–6)

AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, International normalized ratio; IR, Interquartile range.

Twenty three out of 30 (77%) leptospirosis patients were men, and the median age was 48.5 years (range from 19 to 70). The median duration of symptoms before hospital admission was 6 days (range from 4 to 13). The median platelet count and serum creatinine levels were 54 000 cells/mm^3 (range from 10 000 to 222 000) and 6.1 mg/dL (range from 0.6 to 14.2), respectively. All patients required mechanical ventilation, which was performed for a median duration of 2 days (range from 0 to 13). Dialysis was performed in 19 out of 30 (63%) cases. The median duration of hospital stay was 3 days (range from 1 to 6) (Table 1).

The LPHS patients (n = 30) differed from control pulmonary haemorrhage patients (n = 7) in several features: the presence of moderate to high levels of macrophages in the alveolar space (77% vs. 29%, respectively; p 0.02), the presence of the focal hyaline membrane on the alveolar surface (100% vs. 0%; p <0.01), extensive necrosis and regeneration of pneumocyte II cells (100% vs. 0%; p <0.01) and the presence of plasma cells in the alveolar septum (77% vs. 29%, respectively; p 0.02) (Table 2).

No statistically significant differences were observed in the number of other cells in the alveolar septae between LPHS and control patients. Intact leptospires were rarely detected in the lungs of LPHS patients. Reactive debris of granular material associated with macrophages cells was occasionally detected. Leptospiral antigen was not correlated with the intensity of the lesions. None of the patients showed microscopic evidence of disseminated intravascular coagulation.

Lung tissues from 30 LPHS patients and seven controls were examined for the presence of IgM, IgG, IgA and the complement component C3. Three staining patterns were observed for each immunoglobulin and C3 in the lung tissue from the LPHS patients (Fig. 1): AS, delicate linear staining adjacent to the alveolar surface, which was indicative of a membrane covering the luminal surface of type I and II pneumocyte cells; S), heterogeneous staining which was sporadically distributed along the alveolar septum; and IA, weak, focal intra-alveolar granular staining. As shown in Fig. 1, AS-type staining was present in lung tissue samples with diffuse alveolar haemorrhage. This was the most frequent staining pattern observed (12/17, 71%). S-type staining and a mix of S- and AS-type staining were observed in tissues showing mild and/or moderate alveolar haemorrhage (5/17, 29%). The S type was not predominantly associated with either capillaries or epithelial basal membranes.

Out of the 30 LPHS lung tissue samples, 18 were positive for IgA, 17 for IgM, 16 for IgG, and 17 for C3. All 17 C3-positive samples were also positive for at least two Ig classes (Table 2).

Patients with more recent pulmonary haemorrhage demonstrated a higher frequency of C3 and IgG depositions (11/14 and 10/14, respectively) than patients who presented with pulmonary haemorrhage with phagocytosis of haemosiderin pigment (6/16 and 6/16, respectively) (p 0.02) (data not shown).

After separating LPHS patients into two groups based on the median duration of mechanical ventilation, we found that, in patients who used mechanical ventilation for 2 days or less, there was a lower frequency of specimens with macrophages in the alveolar lumen than in patients who were on mechanical ventilation for more than 2 days (31% vs. 80%, p 0.04).

Discussion

LPHS is the major cause of death in patients with leptospirosis. The case fatality rate in patients with LPHS in Brazil ranges from 55 to 74% [5,7]. Understanding the pathogenesis of this disease would thus provide a basis for designing effective therapeutic interventions and allow the identification of biomarkers related to the severity of leptospirosis. The present study suggests that membranous depositions of linear immunoglobulins (IgA, IgG, IgM) and complement on an alveolar surface may trigger fatal pulmonary haemorrhage in human leptospirosis.

TABLE 2. Clinical and microscope characteristics of 30 fatal cases of proven human leptospirosis

						Alveolar space			IHC			
Number	Age	Gender	MV	PI/II	RPH	M	N	L	IgA	IgM	IgG	C3
2	70	M	0	+	+	3	0	1	+	+	+	+
3	33	M	8	+	–	3	1	1	–	–	–	–
5	59	M	5	+	–	2	1	1	–	–	–	–
7	54	M	2	+	–	2	0	1	–	–	–	–
8	20	M	3	+	+	2	1	1	+	+	+	+
10	52	M	6	+	–	2	0	1	+	+	+	+
11	31	M	1	+	+	1	0	1	+	+	–	+
13	58	M	4	+	–	2	1	1	–	–	–	–
17	22	M	7	+	–	3	2	1	–	–	–	–
18	22	F	0	+	+	1	0	1	+	+	+	+
19	30	M	5	+	+	2	0	1	+	+	+	+
20	69	M	1	+	+	2	0	1	+	+	+	+
21	56	M	0	+	+	2	1	1	+	+	+	+
22	63	M	2	+	–	3	1	1	–	–	–	–
23	19	F	0	+	–	1	0	1	IC	IC	IC	IC
24	27	F	0	+	–	2	0	1	IC	IC	IC	IC
27	56	M	0	+	+	2	1	1	+	+	+	+
28	48	F	0	+	+	1	0	1	+	–	–	–
32	21	M	1	+	+	1	1	1	+	+	+	+
34	40	M	0	+	+	1	0	0	IC	IC	IC	IC
35	55	F	1	+	+	1	1	1	+	+	+	+
36	53	F	10	+	–	3	1	1	+	+	+	+
37	54	M	0	+	–	2	0	1	–	–	–	–
38	44	M	2	+	+	2	1	1	IC	IC	IC	IC
42	40	M	8	+	–	2	0	1	+	+	+	+
43	35	F	2	+	–	2	1	1	+	+	+	+
44	40	M	13	+	–	2	1	1	–	–	–	–
45	53	M	0	+	–	3	1	1	+	+	+	+
46	49	M	0	+	+	2	0	1	+	+	+	+
47	59	M	0	+	–	2	0	1	+	+	+	+

M, male; F, female; +, positive; –, negative; IC, inconclusive; MV, days of mechanical ventilation; PI/II, type I and II pneumocyte cells necrosis and regeneration; RPH, rRecent pulmonary haemorrhage; M, N, and L, semi-quantitative presence of macrophages, lymphocytes and neutrophils in the alveolar space.

Immunoglobulin deposits were detected in 18/30 LPHS patients, but were not detected in any of the seven control haemorrhagic lung samples. In all 17 cases in which C3 deposition was present, there was also deposition of IgM, IgG and IgA. The most common staining pattern observed was a linear deposition adjacent to the alveolar surface. The S-type staining was less commonly observed and may have reflected the initial event that resulted in immunoglobulin and complement deposition on the alveolar surface. Lung tissue from leptospirosis and control patients displayed IA-type staining, which was most likely related to the non-specific immunoglobulin and C3 staining in the alveolar space that was filled by cells and plasma components.

The linear deposit pattern (AS-type staining) appeared to cover type I and II pneumocyte cells, leading to the hypothesis that such deposits could interfere with the epithelial barrier. The injury to, and loss of epithelial integrity in, type I and II cells disrupt normal epithelial fluid transport and impair the removal of oedema fluid from the alveolar space [23]. Moreover, injury to type II cells could reduce the production and turnover rate of surfactant, as well as reduce the capacity of oxygen and carbonic gas exchange [24]. Together, these events could result in diffuse alveolar haemorrhage.

Human LPHS is associated with unique histological patterns that differ from other causes of pulmonary haemorrhage such as bleeding diathesis, venous hypertension, other infections, tumour, collagen diseases, pulmonary haemorrhagic syndrome and diffuse alveolar damage (DAD) [25]. The pathological diagnosis for acute-phase DAD is based on the presence of hyaline membranes associated with intra-alveolar red cells and neutrophilis [26]. The LPHS patients, in addition to having immunoglobulin and complement deposition, also displayed pathological characteristics of DAD [27].

Little information regarding the pathological mechanism of LPHS is available. Some clinical leptospirosis manifestations are related to immunological responses such as in uveitis, arthritis and aseptic meningitis [28–31]. Four previous studies have demonstrated the presence of anticardiolipin antibodies in leptospirosis patients, and one series of cases found a correlation between the presence of these antibodies in the sera and severe complications from leptospirosis [32–35]. Finally, Abdulkader *et al.* [36] demonstrated that anti-*Leptospira* antibody levels are likely to be positively correlated with pulmonary haemorrhage.

Recently, Nally *et al.* [16] validated a guinea pig model for LPHS studies. They suggested that the pathogenic mechanisms involved in LPHS best fit with a model of linear immu-

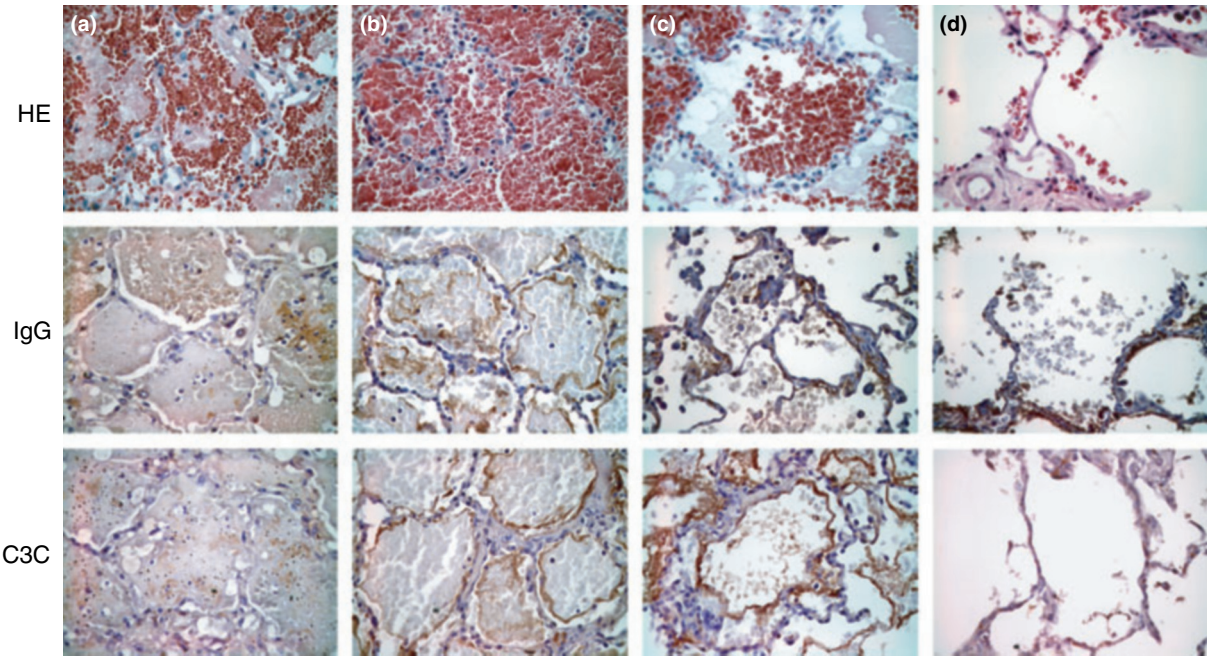

FIG. 1. Alveolar haemorrhage in leptospirosis pulmonary haemorrhage syndrome (LPHS) and control patients, as detected by histology and immunohistochemistry in the alveolar area (Magnification, 400×). (a) Lung from control patients with diffuse alveolar haemorrhage (HE) showing an absence of immunoglobulin and C3 deposition; (b) Lung from LPHS patients showing diffuse alveolar haemorrhage (HE) and a delicate linear immunoglobulin and C3 deposition that is adjacent to the alveolar surface (AS staining pattern); (c) Lung from LPHS patients showing alveolar haemorrhage (HE) and mixed AS and S staining pattern; and (d) Lung from LPHS patients (HE) showing haemorrhage and random, multifocal staining along the alveolar septum (S staining pattern). HE = haematoxylin & eosin.

noglobulin and complement deposition along the alveolar septum as occurs in Goodpasture's syndrome or anti- glomerular basement membrane disease. As stated by these authors, electron microscopy studies were unsuccessful in confirming the deposition of immunoglobulins in the capillary alveolar basal membrane. Also, histological examination of renal tissues did not demonstrate any pathological characteristics of Goodpasture's disease [16]. Regarding the involvement of endothelial cells in the pathogenesis of LPHS, human and experimental studies did not find any pathological features such as endothelial cell necrosis, rupture, thrombus or exposed subendothelial collagen that are characteristic of vasculitis [16,17].

In our study, the majority of patient lung samples presented immunoglobulin and complement deposition adjacent to the alveolar surface correlated with intensive necrosis of type I and II cells. Remarkably, it was not associated with the alveolar septum and the alveolar basal membrane. Therefore, the epithelial barrier seems to be more affected than endothelial cells in human LPHS.

Based on these findings, we proposed that LPHS has unique pathological features not seen in other pulmonary haemorrhagic syndromes. We speculated that the initial increase in vascular permeability as a result of endothelial activation [17,37], overproduction of nitric oxide [38,39] and enlarged open junctions [17,40] would permit IgM, IgG and IgA leakage into the alveolar space. The S-type staining could reflect this initial phase of immunoglobulin leakage through the alveolar septum. The immunglobulin deposition against

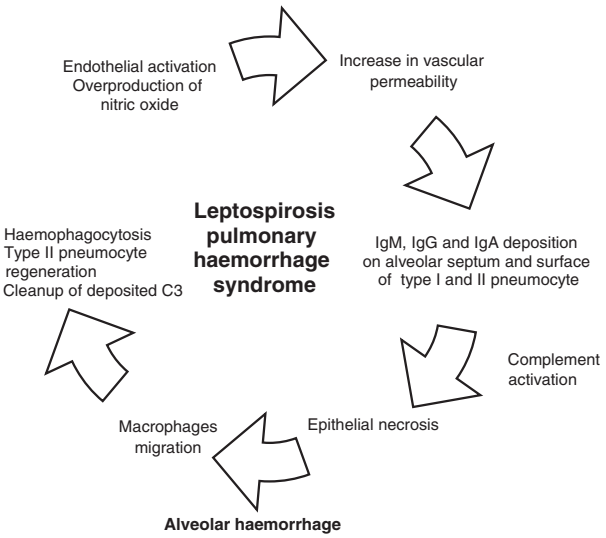

FIG. 2. Proposed pathogenic mechanism involved in leptospirosis pulmonary haemorrhage syndrome (LPHS).

type I and II pneumocyte cells could mediate the complement activation with C3 deposition. This could then lead to epithelial cell necrosis with cell leakage and haemorrhage into the alveolar lumen (Fig. 2). The reasons for the failure to detect C3 in 13 out of the 30 infected lung tissues are unclear, but this may reflect a cleanup of deposited C3 by the macrophage cells in the alveolar lumen, as the absence of C3 deposits correlated with an increase of mononuclear cells in the alveolar lumen in patients with increased mechanical ventilation time. Alternatively, the absence of C3 deposits could be explained by the intrinsically short half-life of C3C [41].

Although it is clear that antibodies and C3 are deposited along the alveolar surface, additional evaluation will be necessary to exclude the possibility that some cellular remnants related to cell activation could cause the non-specific deposition of complement and/or immunoglobulin [17,37,42]. Testing the sera of LPHS patients against normal lung tissues would allow the verification of the specificity of autoantibodies to alveolar components such as type I and II pneumocytes.

Further studies to characterize this apparent autoimmune response and, perhaps, to identify the autoantibody involved, could help to diagnose patients with an increased risk of developing LPHS. Moreover, if an autoimmune process were the cause of haemorrhagic complications in human leptospirosis, effective therapies may involve removing autoantibodies from the blood and decreasing their production.

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Transparency Declaration

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References

1. McBride AJ, Athanazio DA, Reis MG, Ko AI. Leptospirosis. *Curr Opin Infect Dis* 2005; 18: 376–386.
2. Levett PN. Leptospirosis. *Clin Microbiol Rev* 2001; 14: 296–326.
3. Faine S, Adler B, Bolin C, Perolat P. *Leptospira and leptospirosis*, 2nd edn. Melbourne, Australia: MediSci, 1999.
4. Zaki SR, Shieh WJ. Leptospirosis associated with outbreak of acute febrile illness and pulmonary haemorrhage, Nicaragua, 1995. The Epidemic Working Group at Ministry of Health in Nicaragua. *Lancet* 1996; 347: 535–536.
5. Marotto PC, Nascimento CM, Eluf-Neto J *et al.* Acute lung injury in leptospirosis: clinical and laboratory features, outcome, and factors associated with mortality. *Clin Infect Dis* 1999; 29: 1561–1563.
6. Panaphut T, Domrongkitchaiporn S, Thinkamrop B. Prognostic factors of death in leptospirosis: a prospective cohort study in Khon Kaen, Thailand. *Int J Infect Dis* 2002; 6: 52–59.
7. Gouveia EL, Metcalfe J, de Carvalho AL *et al.* Leptospirosis-associated severe pulmonary hemorrhagic syndrome, Salvador, Brazil. *Emerg Infect Dis* 2008; 14: 505–508.
8. Sehgal SC, Murhekar MV, Sugunan AP. Outbreak of leptospirosis with pulmonary involvement in north Andaman. *Indian J Med Res* 1995; 102: 9–12.
9. Yersin C, Bovet P, Merien F *et al.* Pulmonary haemorrhage as a predominant cause of death in leptospirosis in Seychelles. *Trans R Soc Trop Med Hyg* 2000; 94: 71–76.
10. Segura ER, Ganoza CA, Campos K *et al.* Clinical spectrum of pulmonary involvement in leptospirosis in a region of endemicity, with quantification of leptospiral burden. *Clin Infect Dis* 2005; 40: 343–351.
11. Shenoy VV, Nagar VS, Chowdhury AA, Bhalgat PS, Juvale NI. Pulmonary leptospirosis: an excellent response to bolus methylprednisolone. *Postgrad Med J* 2006; 82: 602–606.
12. Tse KC, Yip PS, Hui KM *et al.* Potential benefit of plasma exchange in treatment of severe icteric leptospirosis complicated by acute renal failure. *Clin Diagn Lab Immunol* 2002; 9: 482–484.
13. Yang HL, Jiang XC, Zhang XY *et al.* Thrombocytopenia in the experimental leptospirosis of guinea pig is not related to disseminated intravascular coagulation. *BMC Infect Dis* 2006; 6: 19.
14. Edwards CN, Nicholson GD, Hassell TA, Everard CO, Callender J. Thrombocytopenia in leptospirosis: the absence of evidence for disseminated intravascular coagulation. *Am J Trop Med Hyg* 1986; 35: 352–354.
15. Nicodemo AC, Del Negro G, Amato Neto V. Thrombocytopenia and leptospirosis. *Rev Inst Med Trop Sao Paulo* 1990; 32: 252–259.
16. Nally JE, Chantranuwat C, Wu XY *et al.* Alveolar septal deposition of immunoglobulin and complement parallels pulmonary hemorrhage in a guinea pig model of severe pulmonary leptospirosis. *Am J Pathol* 2004; 164: 1115–1127.
17. Nicodemo AC, Duarte MI, Alves VA, Takakura CF, Santos RT, Nicodemo EL. Lung lesions in human leptospirosis: microscopic, immunohistochemical, and ultrastructural features related to thrombocytopenia. *Am J Trop Med Hyg* 1997; 56: 181–187.
18. Silva JJ, Dalston MO, Carvalho JE, Setubal S, Oliveira JM, Pereira MM. Clinicopathological and immunohistochemical features of the severe pulmonary form of leptospirosis. *Rev Soc Bras Med Trop* 2002; 35: 395–399.
19. Yang GG, Hsu YH. Nitric oxide production and immunoglobulin deposition in leptospiral hemorrhagic respiratory failure. *J Formos Med Assoc* 2005; 104: 759–763.
20. Alves VA, Vianna MR, Yasuda PH, De Brito T. Detection of leptospiral antigen in the human liver and kidney using an immunoperoxidase staining procedure. *J Pathol* 1987; 151: 125–131.
21. Alves VA, Yasuda PH, Yamashiro EH, Santos RT, Yamamoto LU, de Brito T. An immunohistochemic assay to localize leptospires in tissue specimens. *Rev Inst Med Trop Sao Paulo* 1986; 28: 170–173.
22. Moussallem TM, Guedes F, Fernandes ER *et al.* Lung involvement in childhood measles: severe immune dysfunction revealed by quantitative immunohistochemistry. *Hum Pathol* 2007; 38: 1239–1247.

23. Andrade L, Rodrigues AC Jr, Sanches TR, Souza RB, Seguro AC. Leptospirosis leads to dysregulation of sodium transporters in the kidney and lung. *Am J Physiol Renal Physiol* 2007; 292: f586–f592.
24. Castranova V, Rabovsky J, Tucker JH, Miles PR. The alveolar type II epithelial cell: a multifunctional pneumocyte. *Toxicol Appl Pharmacol* 1988; 93: 472–483.
25. Mills SE. *Sternberg's diagnostic surgical pathology*. Philadelphia: Lippincott Williams & Wilkins, 2004.
26. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2004; 342: 1334–1349.
27. Blennerhassett JB. Shock lung and diffuse alveolar damage pathological and pathogenetic considerations. *Pathology* 1985; 17: 239–247.
28. Rathinam SR. Ocular manifestations of leptospirosis. *J Postgrad Med* 2005; 51: 189–194.
29. Finsterer J, Stollberger C, Sehnal E, Stanek G. Mild leptospirosis with three-year persistence of IgG- and IgM-antibodies, initially manifesting as carpal tunnel syndrome. *J Infect* 2005; 51: E67–E70.
30. Silva HR, Tanajura GM, Tavares-Neto J *et al.* [Aseptic meningitis syndrome due to enterovirus and Leptospira sp in children of Salvador, Bahia]. *Rev Soc Bras Med Trop* 2002; 35: 159–165.
31. Sperber SJ, Schleupner CJ. Leptospirosis: a forgotten cause of aseptic meningitis and multisystem febrile illness. *South Med J* 1989; 82: 1285–1288.
32. Rugman FP, Pinn G, Palmer MF, Waite M, Hay CR. Anticardiolipin antibodies in leptospirosis. *J Clin Pathol* 1991; 44: 517–519.
33. Santiago M, Martinelli R, Reis MG *et al.* Diagnostic performance of anti-beta2 glycoprotein I and anticardiolipin assays for clinical manifestations of the antiphospholipid syndrome. *Clin Rheumatol* 2004; 23: 485–489.
34. Tattevin P, Dupeux S, Hoff J. Leptospirosis and the antiphospholipid syndrome. *Am J Med* 2003; 114: 164.
35. De Francesco Daher E, Oliveira Neto FH, Ramirez SM. Evaluation of hemostasis disorders and anticardiolipin antibody in patients with severe leptospirosis. *Rev Inst Med Trop Sao Paulo* 2002; 44: 85–90.
36. Abdulkader RC, Daher EF, Camargo ED, Spinosa C, da Silva MV. Leptospirosis severity may be associated with the intensity of humoral immune response. *Rev Inst Med Trop Sao Paulo* 2002; 44: 79–83.
37. Gomez RM, Vieira ML, Schattner M *et al.* Putative outer membrane proteins of Leptospira interrogans stimulate human umbilical vein endothelial cells (HUVECS) and express during infection. *Microb Pathog* 2008; 45: 315–322.
38. Maciel EA, Athanazio DA, Reis EA *et al.* High serum nitric oxide levels in patients with severe leptospirosis. *Acta Trop* 2006; 100: 256–260.
39. Hsu YH, Chen HI. The involvement of nitric oxide and beta-adrenergic pathway signalling in pulmonary oedema and fluid clearance. *Pathology* 2007; 39: 612–613.
40. De Brito T, Bohm GM, Yasuda PH. Vascular damage in acute experimental leptospirosis of the guinea-pig. *J Pathol* 1979; 128: 177–182.
41. Seifert PS, Messner M, Roth I, Bhakdi S. Analysis of complement C3 activation products in human atherosclerotic lesions. *Atherosclerosis* 1991; 91: 155–162.
42. Vieira ML, D'Atri LP, Schattner M *et al.* A novel leptospiral protein increases ICAM-1 and E-selectin expression in human umbilical vein endothelial cells. *FEMS Microbiol Lett* 2007; 276: 172–180.